REMARKS

In the Office Action dated November 16, 2004, Claims 1-25 are pending in the application. Claims 1-19, 21, 22, 24 and 25 have been withdrawn from consideration. Claims 20 and 23 are under examination. Claims 20 and 23 are rejected under 35 U.S.C. §101. Claim 23 remains rejected under 35 U.S.C. §112, second paragraph. Claims 20 and 23 are rejected 35 U.S.C. §102(b) as allegedly anticipated by Kamura et al. (*Genes and Development* 12: 3872-3881, 1998). Claims 20 and 23 are further rejected under 35 U.S.C. §103(a) as allegedly unpatentable over Kamura et al. In addition, the specification is objected to for allegedly failing to comply with the Sequence Rules.

This Response addresses each of the Examiner's rejections. Applicants therefore respectfully submit that the present application is in condition for allowance. Favorable consideration of all pending claims is therefore respectfully requested.

The Examiner has objected to the specification for referencing "SPRY" without a sequence identifier. Further, the Examiner indicates that Applicants did not include a statement in the previous Response that the Sequence Listing submitted does not introduce new matter.

In response, Applicants are providing herewith a substitute Sequence Listing, which includes a new sequence identifier, SEQ ID NO: 6, which sets forth the peptide sequence of SPRY. Applicants have also amended the specification to direct the entry of SEQ ID NO: 6. Furthermore, Applicants are providing a Statement under 37 C.F.R. §1.821(f), verifying the identity of the paper copy and the computer-readable copy of the Sequence Listing and stating that the substitute Sequence Listing does not introduce new matter. Thus, the objection to the specification is overcome and withdrawal thereof is respectfully requested.

Claims 20 and 23 are rejected under 35 U.S.C. §101. The Examiner alleges that the claimed invention is not supported by either a specific and substantial asserted utility or a well-

established utility. Further, Claims 20 and 23 are rejected under 35 U.S.C. §112, first paragraph. The Examiner states that since the claimed invention is not supported by either a specific and substantial asserted utility or a well-established utility, one skilled in the art would not know how to use the claimed invention.

According to the Examiner, the purpose of the claimed methods is to either inhibit or promote protein degradation by modulating the interaction between an SOCS box and elongin C. However, the Examiner is of the opinion that the claimed methods have no apparent or disclosed utility, and that Applicants have not provided the utility of the proteins that comprise a SOCS box. Further, the Examiner questions the effects of inhibiting or promoting the degradation of all the proteins that meet the limitations of these claims.

Applicants respectfully submit that the present invention is predicated in part on the demonstration that the SOCS-box in proteins facilitates the presentation of proteins to the ubiquitination and/or proteasomal compartment. Accordingly, the SOCS-box containing protein forms a family of adaptor proteins which terminate cell signaling by targeting critical molecules for intracellular degradation. This unique recognition by the present inventors permits the development of a range of molecules that are capable of modulating SOCS proteins. Claims 20 and 23 are directed towards methods for identifying an agonist of the elongin-SOCS box interaction, as well as an antagonist of this interaction. An agonist promotes degradation of a cytokine and hence terminates cytokine-mediated signaling. On the other hand, an antagonist inhibits degradation of a cytokine and promotes continued signaling.

As disclosed in the specification, e.g., page 12, lines 14-15, it may be desirable to have the continuation of signaling in various circumstances such as the treatment of cancer or other disorders. In other circumstances, however, it may desirable to terminate signaling by targeting critical signaling molecules for intracellular degradation. See page 12, lines 6-16 of the

specification. Signaling molecules that have been shown to bind to SOCS-1 (a SOCS box-containing protein) include JAK1 and gp130 (page 36, lines 18-24). Applicants respectfully submit that modulation of cytokine signaling clearly has a specific and substantial utility or a well-established utility.

In support of Applicant's position, Applicants provide herewith a paper by Dr. Jian Guo Zhang and colleagues from the Walter and Eliza Hall Institute of Medical Research, entitled "The SOCS-box of suppressor of cytokine signaling-1 is important for inhibition of cytokine action *in vivo*", published in *PNAS* 98 (23): 13261-13265, 2001 (**Exhibit 1**). This article refers specifically to SOCS-1 and demonstrates that it is an essential physiological inhibitor of IFN-γ signaling. Mice that lack the SOCS-1 gene die in the early postnatal period from a disease characterized by hyper-responsiveness to endogenous IFN-γ. Dr. Zhang and his colleagues have shown that the mere deletion of the SOCS-box from the SOCS-1 protein leads to partial loss of function of the SOCS-1 protein, increased responsiveness of the mice to IFN-γ, and development of a fatal inflammatory disease. Consequently, the authors conclude that the SOCS-box is an important element in the SOCS-1 protein and it contributes to inhibition of cytokine signaling.

Applicants are also providing herewith a manuscript by Dr. Marlyse Debrincat and colleagues from the Walter and Eliza Hall Institute of Medical Research Institute, entitled "Ankyrin Repeat and SOCS Box Containing Protein Asb-9 Targets Creatine Kinase B for Degradation" (**Exhibit 2**). As stated in the summary of the manuscript, the SOCS proteins inhibit cytokine signaling by direct interaction with Janus kinases (JAKs) or via activated cytokine receptors. Dr. Marlyse Debrincat employed a proteomic approach and demonstrated that targeting of creatine kinase B for degradation by Asb-9 was entirely SOCS-box dependent. It should be recognized that Asb-9 is a protein containing a SOCS-box, and that creatine kinase

is critical for the maintenance and regulation of cellular energy stores in tissues with high and rapidly-changing energy demands, such as brain and skeletal and cardiac muscle.

Applicants respectfully submit that the article by Dr. Zhang and the manuscript by Dr. Debrincat further support the notion that SOCS-box containing proteins modulate degradation of cytokines and therefore the signaling mediated by cytokines. In view of the such supporting showing and the teaching in the present specification, it is apparent to those skilled in the art that the claimed methods are useful for identifying an agonist or antagonist of the interaction between an SOCS box and elongin, which can then be employed in modulating cytokine signaling. As submitted above, it is clear to those skilled in the art that modulation of cytokine signaling has a specific and substantial utility or a well-established utility.

Accordingly, it is respectfully submitted that the claimed methods fully comply with the utility requirement under 35 U.S.C. §101. Withdrawal of the rejections under 35 U.S.C. §101 and §112, first paragraph, is therefore respectfully requested.

Claim 23 is rejected under 35 U.S.C. §112, second paragraph, as allegedly indefinite. It is respectfully submitted that claim 23, a presently amended, is not indefinite. Withdrawal of the rejection is therefore respectfully requested.

Claims 20 and 23 are rejected under 35 U.S.C. §102(b) as allegedly anticipated by Kamura et al. (*Genes and Development* 12: 3872-3881, 1998). Claims 20 and 23 are also rejected under 35 U.S.C. §103(a) as allegedly unpatentable over Kamura et al.

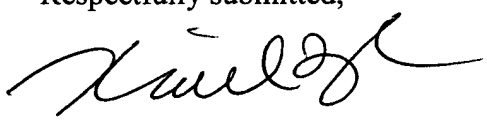
The Examiner contends that the claimed invention is directed to decreasing SOCS protein expression by promoting its degradation through a SOCS box/elongin C complex. According to the Examiner, Kamura et al. teach that a SOCS box/elongin C complex increases the expression of SOCS proteins by inhibiting its degradation. The Examiner contends that although Kamura et al. do not specifically teach that a SOCS box/elongin complex decreases

SOCS protein expression by promoting degradation, the skilled artisan would immediately envision this based on the teaching of Kamura, et al. The Examiner reasons that if increased expression occurs via inhibiting degradation, then decreased expression must occur via promoting degradation.

Applicants respectfully submit that rather than disclosing the claimed invention, Kamuara et al. teach the opposite of the claimed invention. In particular, Kamura et al. teach that a SOCS box/elongin C complex increases the expression of SOCS proteins by inhibiting its degradation. This teaching of Kamuara et al. indicates that, as a result of the interaction between the SOCS box and elongin C, the *stability* of SOCS proteins is *improved.* In contrast, the present invention is premised on the recognition that the SOCS-box is essential to the *degradation* of SOCS box-containing proteins in a proteasome-dependent manner. That is, according to the present invention and contrary to the teaching of Kamuara et al., the interaction between the SOCS box and elongin C confers *instability* on SOCS box-containing proteins. Therefore, the claimed invention is not taught or rendered obvious by Kamuara et al. Withdrawal of the rejections based on Kamuara et al. is therefore respectfully requested.

In view of the foregoing amendments and remarks, it is firmly believed that the subject application is in condition for allowance, which action is earnestly solicited.

Respectfully submitted,



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The SOCS box of suppressor of cytokine signaling-1 is important for inhibition of cytokine action *in vivo*

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Contributed by Donald Metcalf, September 17, 2001

Suppressor of Cytokine Signaling-1 (SOCS-1) is an essential physiological inhibitor of IFN-γ signaling. Mice lacking this gene die in the early postnatal period from a disease characterized by hyperresponsiveness to endogenous IFN-γ. The SOCS box is a C-terminal domain shared with over 30 other proteins that links SOCS proteins to an E3 ubiquitin ligase activity and the proteasome, but whether it contributes to inhibition of cytokine signaling is currently disputed. We have deleted only the SOCS box of the SOCS-1 gene in mice and show that such mice have an increased responsiveness to IFN-γ and slowly develop a fatal inflammatory disease. These results demonstrate that deletion of the SOCS box leads to a partial loss of function of SOCS-1.

Suppressor of Cytokine Signaling-1 (SOCS-1) was discovered independently by three groups and shown to be an important negative-feedback inhibitor of cytokine-activated Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathways (1–3). SOCS-1 contains an SH2 domain that recognizes activated JAKs and a kinase-inhibitory region that is proposed to inhibit JAK Tyr kinase activity (4–6). Mice lacking the SOCS-1 gene die with fatty degeneration and necrosis of the liver before weaning and a T-cell-dependent multiorgan inflammatory disease. These mice display hypersensitivity to IFN-γ, and mice lacking both SOCS-1 and IFN-γ fail to develop the postnatal disease seen in SOCS-1$^{-/-}$ mice (7–9).

SOCS-1 also contains a C-terminal domain with homology to the previously defined cytokine-inducible SH2-containing protein (CIS). We termed this homology domain the SOCS box (1). Subsequently, we and others have defined a large family of proteins that contain a C-terminal SOCS box and either an SH2 domain (CIS and SOCS-1 to SOCS-7), ankyrin repeats (ASB-1 to ASB-18), WD40 repeats (WSB-1 and -2 as well as Tubby-like protein), a SPRY domain (SSB-1 to SSB-4), a small GTPase (RAB) domain (4 RAR-like proteins), or a neuralized domain (1 protein) (10–13). The SOCS box from several family members was shown to act as an independent binding domain for the elongin B/C complex (14, 15) that, in turn, is part of an E3 ubiquitin ligase complex (16). The same complex also has been shown to bind to the Von Hippel–Lindau tumor-suppressor protein (that also contains a SOCS box-like sequence) and to induce proteasomal degradation of bound hypoxia-inducible transcription factors (17). Recent *in vitro* studies have demonstrated SOCS-1-dependent ubiquitination and proteasomal degradation of vav and the TEL/JAK2 fusion protein; in the latter case, at least, this ubiquitination depends on an intact SOCS box (18–20). Despite this observation, functional studies of SOCS proteins have generally failed to demonstrate a requirement of the SOCS box for inhibition of cytokine signaling *in vitro* (4–6), and two studies have suggested that the SOCS box instead stabilizes SOCS proteins by inhibiting their proteasomal degradation (5, 14). We have addressed the physiological role of the SOCS box by deleting only this element from the SOCS-1 gene in mice and comparing their phenotype to that of mice lacking the entire SOCS-1 protein.

Materials and Methods

Generation of SOCS-1 Δ/Δ Mice. The murine SOCS-1 gene is shown in Fig. 2. A nested PCR protocol was used to amplify the 2.9-kb *Bam*HI exon-bearing fragment from the SOCS-1 gene that lacked the sequence encoding the C-terminal 41 aa before the translation-termination codon. A 3.2-kb *Bam*HI–*Eco*RV fragment situated immediately 3′ also was isolated, and these two fragments were inserted upstream and downstream of a loxP-flanked PGKneo cassette to produce the targeting vector. This vector was linearized and electroporated into C57BL/6 embryonic stem (ES) cells, and after selection in 175 μg/ml G418, resistant clones were picked and screened. *Hin*dIII-digested genomic DNA isolated from individual clones was hybridized with a 0.7-kb *Bam*HI–*Nhe*I fragment located upstream of sequences in the targeting vector to identify clones in which homologous recombination had occurred. This strategy provided distinction between the endogenous (~20-kb) and targeted (4.5-kb) SOCS-1 alleles. An ES-cell clone, in which an endogenous SOCS-1 allele had undergone homologous recombination as confirmed by *Hin*dIII restriction endonuclease analysis and DNA sequencing, was expanded and injected into BALB/c blastocysts to generate chimeric mice. Male chimeras were mated with C57BL/6 females to yield heterozygotes for the targeted SOCS-1 allele, which were interbred to produce wild-type (+/+), heterozygous (Δ/+) and homozygous SOCS box-deleted mutant (Δ/Δ) mice on a pure C57BL/6 genetic background. SOCS-1$^{+/+}$IFN-γ$^{-/-}$, SOCS-1$^{\Delta/\Delta}$IFN-γ$^{-/-}$, and SOCS-1$^{+/\Delta}$IFN-γ$^{-/-}$ mice were generated by crossing SOCS-1$^{\Delta/+}$ mice with C57BL/6 IFN-γ$^{-/-}$ mice and then intercrossing SOCS-1$^{+/\Delta}$IFN-γ$^{+/-}$ offspring.

Immunoprecipitation and Western Blotting. For analysis of SOCS-1 proteins in mouse organs, tissue extracts were prepared from whole thymus, 80 mg of spleen, 200 mg of liver, and 130 mg of lung tissue 4 h after injection of mice with 5 μg of IFN-γ. SOCS-1 and SOCS-1/ΔSB proteins from each tissue extract were immunoprecipitated with 3 μg of anti-SOCS-1 monoclonal antibody and Western blotted with 1 μg/ml of an independent biotinylated anti-SOCS-1 antibody (4H1).

For signal-transduction studies, adult mice weighing an average of 29 g were given a single i.p. injection of either 0.2 ml of saline or 2 μg of rmIFN-γ (PeproTech, Rocky Hill, NJ) in 0.2 ml of saline. Livers were dissected at the indicated times after injection, immediately frozen on dry ice, and stored at −70°C. Frozen livers were homogenized in RIPA buffer [1% (vol/vol) Triton X-100/1% (wt/vol) sodium deoxycholate/0.1% (wt/vol) SDS/150 mM NaCl/10 mM Tris, pH 7.5] containing 10 μg/ml

Abbreviations: SOCS-1, suppressor of cytokine signaling-1; JAK, Janus kinase; STAT, signal transducer and activator of transcription; CIS, cytokine-inducible SH2-containing protein.

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leupeptin (Auspep, Parkville, Australia), 1 mM iodoacetic acid, 50 μg/ml soybean trypsin inhibitor, 20 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM EGTA (Sigma), and 1 mM pervanadate. Pervanadate was prepared by adding hydrogen peroxide (20 mM final) to 1 mM sodium orthovanadate (Sigma) and by incubating at room temperature for 30 min. Lysates were cleared by centrifugation, and protein content was quantitated by using a Coomassie Protein Assay Reagent (Pierce). For immunoprecipitations, liver lysates were incubated with 4 μg of anti-STAT1 antibody (Transduction Laboratories, Lexington, KY) for 30 min on ice and then with 30 μl of 50% protein G Sepharose slurry for 2 h or overnight. After repeated washing in RIPA buffer, bound proteins were eluted from the protein G Sepharose in 40 μl of 2× SDS loading buffer [125 mM Tris·HCl, pH 6.8/2% (wt/vol) SDS/20% (vol/vol) glycerol/100 mM DTT/0.01% bromophenol blue] at 95°C for 2 min, centrifuged at 14,800 × *g* for 5 min, and the supernatant then was loaded on Bio-Rad Criterion SDS/7.5% PAGE gels. Proteins were fractionated by SDS/PAGE under reducing conditions and then electrophoretically transferred to PVDF-Plus membranes (Micron Separations, Westborough, MA). Membranes were blocked with 2% (vol/vol) BSA, 0.1% Tween 20 in phosphate-buffered saline for a minimum of 1 h, and then incubated overnight with anti-phospho-STAT1 antibody (1:1,000, New England Biolabs). Antibody binding was visualized with horseradish peroxidase-conjugated anti-rabbit antibody (Silenus, Paris) and the SuperSignal West Pico chemiluminescent substrate (Pierce).

Hepatocyte Cultures. Hepatocytes were harvested from 10-week-old mice as described (21). Briefly, livers were perfused retrogradely through the inferior vena cava with Hanks' balanced salt solution (HBSS) without calcium and magnesium and then with the same medium containing 0.5 mM EDTA. EDTA then was removed by flushing the liver with HBSS without calcium and magnesium. Hepatocytes were released by perfusing with HBSS without magnesium but with 5 mM $CaCl_2$ and 0.05% collagenase IV (Sigma). Viable hepatocytes were purified by using a Percoll density gradient (Amersham Pharmacia), and the resulting cell pellet was washed three times by 50 × *g* centrifugation in RPMI 1640 medium containing 10% (vol/vol) FCS and 50 μM 2-mercaptoethanol (RF10 medium). Cells were plated in 175-cm^2 culture flasks (Corning) in the same medium at a density of 5 × 10^6 cells per flask and were allowed to adhere for 2 h at 37°C.

Results and Discussion

In previous studies, we demonstrated that deletion of the SOCS box abolished the capacity of SOCS-1 to interact with elongins B and C (15). To investigate the capacity of the SOCS box-deleted form of SOCS-1 (SOCS-1/ΔSB) to interact with JAK kinases, we stably expressed FLAG epitope-tagged SOCS-1 or SOCS-1ΔSB in M1 leukemic cells. The SOCS-1 proteins were purified from cellular extracts by using anti-FLAG antibody chromatography and were analyzed by Western blotting with anti-JAK1 and anti-FLAG antibodies. These studies revealed copurification of JAK1 with both SOCS-1 and SOCS-1/ΔSB (Fig. 1), indicating that the deletion of the SOCS box did not disrupt the capacity of SOCS-1 to bind JAK1.

Mice that contained a homozygous deletion of the SOCS box in the SOCS-1 gene (Δ/Δ mice expressing SOCS-1/ΔSB protein) were generated as indicated in Fig. 2. The induction and expression levels of SOCS-1 or SOCS-1/ΔSB proteins were assessed in the thymus, spleen, liver, and lung of wild-type, Δ/+, and Δ/Δ mice 4 h after injection of IFN-γ (Fig. 2*c*). The data showed that Δ/Δ mice produced a truncated SOCS-1 protein of the expected molecular weight for a molecule lacking the SOCS box. The levels of SOCS-1/ΔSB protein in different tissues from Δ/Δ mice were similar to those of SOCS-1 protein in wild-type

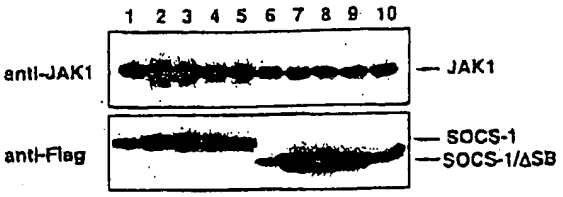


Fig. 1. Interaction of SOCS-1ΔSB with JAK1. Cellular extracts from M1 cells stably expressing either N-terminally Flag-tagged SOCS-1 or SOCS-1 lacking the SOCS box (SOCS-1/ΔSB) were incubated with anti-Flag antibody M2 resin and bound proteins eluted with Flag peptide. (Lanes 1–5) Column eluates from M1 cells expressing full-length SOCS-1. (Lanes 6–10) Column eluates from M1 cells expressing SOCS-1/ΔSB.

mice. Curiously, in Δ/+ heterozygotes only, SOCS-1/ΔSB protein was present at significantly lower levels than the wild-type protein (Fig. 2*c*).

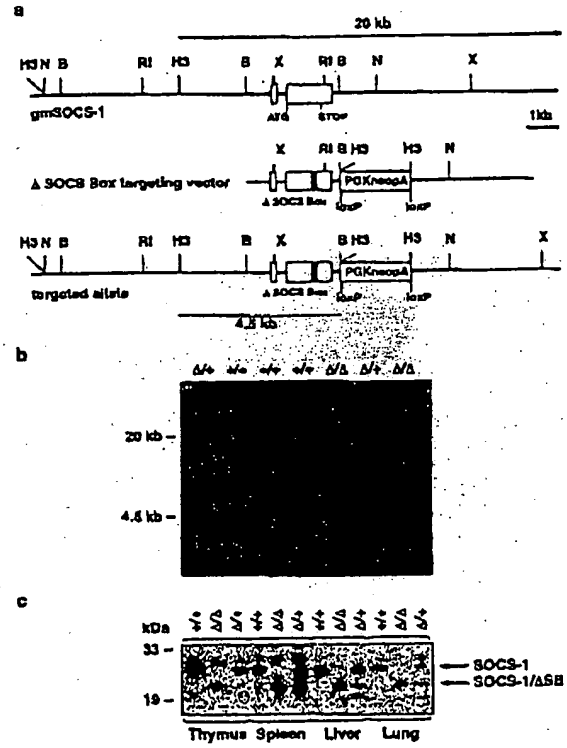


Fig. 2. Generation of Δ/Δ mice through specific deletion of the SOCS box from the SOCS-1 gene by homologous recombination in embryonic stem cells. (*a*) The murine SOCS-1 gene is shown (B, *Bam*HI; N, *Nde*I; X, *Xba*I; RI, *Eco*RI; H3, *Hin*dIII) with the exons represented by open boxes and the deleted SOCS box shown by shaded boxes. (*b*) Southern blot of *Hin*dIII-digested genomic DNA extracted from the tails of mice derived from a cross between heterozygous (Δ/+) mice hybridized with a probe located immediately 5′ to the sequence included in the targeting vector. This distinguishes the 20-kb wild-type genomic fragment from a 4.5-kb targeted allele fragment. (*c*) Western blot showing expression of a smaller SOCS-1 protein consistent with the absence of the SOCS box in various tissues of Δ/Δ mice 4 h after injection with 5 μg of IFN-γ. Extracts were prepared from thymus, spleen, liver, and lung tissue; SOCS-1 and SOCS-1/ΔSB proteins were immunoprecipitated with anti-SOCS-1 monoclonal antibody and then Western blotted with a second biotinylated anti-SOCS-1 antibody. +/+, wild-type mice.

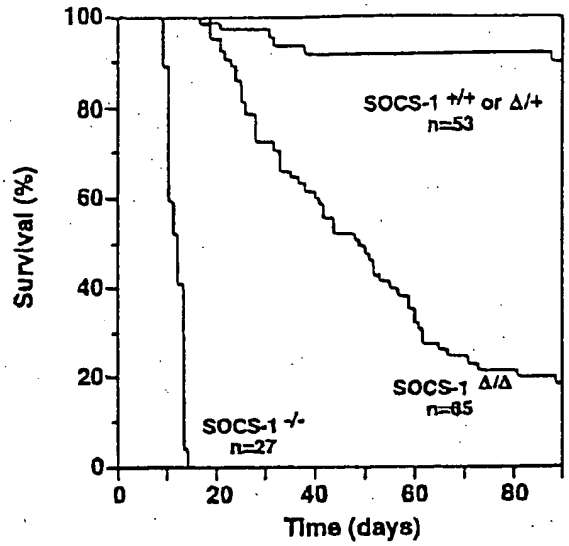
Table 1. Pathology in moribund Δ/Δ mice

Pathology*	Frequency	%
Skeletal muscle–infiltration	18/20	90
Heart–infiltration	18/20	90
Lung–lymphoid foci	12/20	60
–alveolar infiltration	13/20	65
–pneumonia	5/20	25
Pancreas–dispersion and infiltration	11/20	55
Salivary gland–lymphoid foci	11/18	61
Cornea–infiltration	10/19	53
–ulcer	3/19	16
Skin–infiltration of dermis	10/20	50
Marrow–granulocytes > mononuclear cells	10/20	50
Thymus–atrophy or cortical thinning	19/20	95
Liver–trabecular atrophy	8/20	40
–hematopoietic foci	7/20	35
Spleen–no germinal centers	11/20	55
–excess erythropoiesis	11/20	55
Gut–infiltration	3/20	15
Kidney–lymphoid foci	3/20	15
–polycystic disease	1/20	5
–necrosis	1/20	5

*Assessed by histological examination of organs from moribund mice of 40–90 days of age.

Homozygous Δ/Δ mice were healthy at birth but died prematurely compared with Δ/+ or wild-type animals (Fig. 3). However, the onset of disease was prolonged compared with SOCS-$1^{-/-}$ mice, and it most closely matched that seen previously for SOCS-$1^{-/-}$IFN-$\gamma^{+/-}$ mice (22). In fact, the predominant disease states seen in Δ/Δ mice were remarkably similar in frequency and type to those of moribund SOCS-$1^{-/-}$IFN-$\gamma^{+/-}$ mice of the same age (22). Body weight was reduced and extensive inflammatory lesions were seen in skeletal and heart muscle, as well as in the cornea, sometimes with corneal ulceration. The infiltrating cells were predominantly lymphoid cells and macrophages, but the damage to muscle cells was often more severe than that seen in SOCS-$1^{-/-}$IFN-$\gamma^{+/-}$ mice. In addition, lymphoid foci were observed in the salivary glands, lungs, and kidneys of Δ/Δ mice, and the ratio of granulocytes to mononuclear (lymphoid and erythroid) cells was elevated in the bone marrow. Like SOCS-$1^{-/-}$ mice, Δ/Δ mice showed atrophy and dispersion of pancreatic acinar tissue with lymphoid and macrophage infiltration of both the pancreas and dermis and atrophy or thinning of the thymic cortex. Although liver cells did not show fatty degeneration typical of SOCS-$1^{-/-}$ mice, there were areas where the liver trabeculae were thin and widely separated, suggesting previous damage to hepatocytes. The frequencies of these pathologies in Δ/Δ mice are shown in Table 1, and typical sections are shown in Fig. 3. This phenotype suggested that SOCS-1/ΔSB acted as a partial loss of function mutant with significantly reduced capacity to inhibit responses to endogenous IFN-γ.

The response of Δ/Δ mice to IFN-γ was examined by measuring the amount of activated STAT-1 in the liver 4 h after i.v. injection of IFN-γ. STAT-1 Tyr phosphorylation was elevated in Δ/Δ mice compared with wild-type or Δ/+ animals (Fig. 4*a*). The elevated levels of activated STAT-1 in Δ/Δ mice could be caused by the induction of increased amounts or a decreased rate of inactivation of STAT-1. To address this issue and to eliminate the complication of variable levels of endogenous IFN-γ, Δ/Δ mice were generated on an IFN-$\gamma^{-/-}$ genetic background, and the time course of induction and inactivation of activated STAT-1 in the liver in response to injected IFN-γ was measured



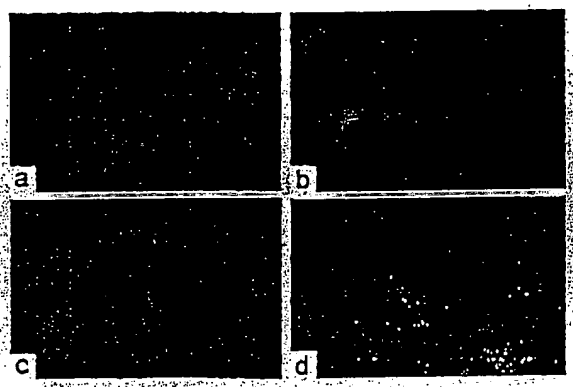


Fig. 3. Disease onset and pathology in mice with deletion of the SOCS box in the SOCS-1 gene. (*Upper*) Survival curves for SOCS-$1^{-/-}$, Δ/Δ, and pooled Δ/+ and +/+ mice on a pure C57BL/6 genetic background (*n* = number of mice). Mice were killed when moribund. (*Lower*) Histology of tissue sections taken from moribund Δ/Δ mice between the ages of 40 and 90 days. (*a*) Skeletal muscle showing extensive cellular infiltration and destruction of muscle cells. (*b*) Heart tissue showing extensive cellular infiltration. (*c*) Cornea with extensive cellular infiltration. (*d*) Lymphoid focus in lung with infiltration of alveolar walls.

(Fig. 4*b*). On an IFN-$\gamma^{-/-}$ background, activated STAT-1 accumulated with similar initial kinetics and to a similar level in both Δ/Δ and Δ/+ animals, but the decline of activated STAT-1 levels to baseline was slowed by at least 2 h in the livers of Δ/Δ mice. In contrast, the levels of full-length SOCS-1 in Δ/+ animals and of SOCS-1/ΔSB in Δ/Δ animals were nearly identical throughout the time course (Fig. 4*c*), but, as was seen in Fig. 2, the levels of SOCS-1/ΔSB protein were again reduced in Δ/+ animals. These data are consistent with the current model in which activated STATs contribute to increasing the transcription of the SOCS-1 gene and the accumulation of SOCS-1 protein. Once produced, SOCS-1 inhibits JAKs, and in doing so, the level of phosphorylated STAT-1 declines. Because the levels of SOCS-1/ΔSB in Δ/Δ liver were similar to those of full-length SOCS-1 in wild-type animals at the 2- to 6-h time points, the data also

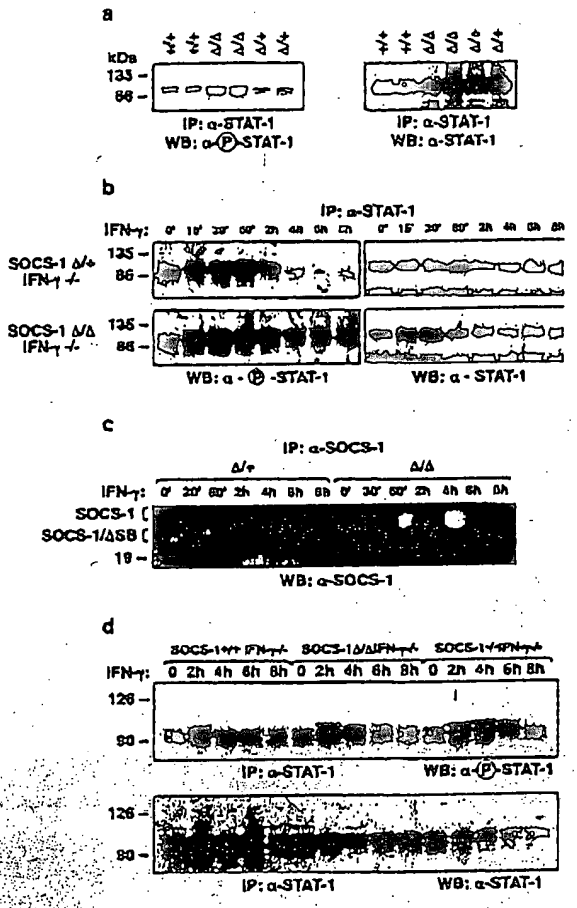


Fig. 4. Response of Δ/Δ mice to IFN-γ. (*a*) Enhanced levels of phosphorylated STAT-1 in livers of Δ/Δ mice 4 h after injection of IFN-γ. Protein was extracted from livers, immunoprecipitated with an anti-STAT-1 monoclonal antibody, and Western blotted either with an antibody specific for Tyr-phosphorylated (activated) STAT-1 (*Left*) or total STAT-1 (*Right*). +/+, wild-type mice; Δ/+, heterozygous mice. (*b*) Time course of STAT-1 activation and deactivation in the liver after a single injection of IFN-γ into Δ/Δ or Δ/+ mice bred onto an IFN-$\gamma^{-/-}$ genetic background. Activated (phosphorylated) STAT-1 (*Left*) and total STAT-1 (*Right*) are shown. Immunoprecipitation and Western blotting were essentially as described for *a*. (*c*) Time course of SOCS-1 or SOCS-1/ΔSB induction and clearance in the livers of the same animals as described in *b*. Immunoprecipitation and Western blotting were essentially as described for Fig. 2*c*. (*d*) Comparison of STAT-1 response to IFN-γ in primary hepatocyte cultures derived from +/+, Δ/Δ, and −/− mice all bred to an IFN-$\gamma^{-/-}$ background. Cultures were stimulated with IFN-γ for 10 min and then harvested at the indicated time points. Immunoprecipitation and Western blotting of activated STAT-1 were performed as described above.

clearly suggest that SOCS-1/ΔSB is defective in terminating signal transduction and hence in reducing the levels of activated STAT-1.

These data were confirmed in isolated hepatocyte cultures from Δ/Δ and +/+ mice on an IFN-$\gamma^{-/-}$ background (Fig. 4*d*), suggesting that this behavior of full-length and truncated SOCS-1 was independent of more complex cellular interactions. Consistent with the disease incidence profiles in Fig. 3, the prolonged activation of STAT-1 seen in hepatocytes of Δ/Δ mice was intermediate between that seen in SOCS-$1^{+/+}$ and SOCS-$1^{-/-}$ mice (Fig. 4*d*). On an IFN-$\gamma^{-/-}$ background, the levels of activated STAT-1 declined by 4 h in hepatocytes from wild-type mice, by 6 h in Δ/Δ mice, and by ≈8 h in SOCS-$1^{-/-}$ mice. This finding suggests that two separate mechanisms are involved in the termination of signal transduction by SOCS-1, one involving the SOCS box and the other involving the SH2 and N-terminal domains. A role for the SH2 and N-terminal domains has already been proposed in which the SH2 domain binds to a phosphorylated Tyr in the JAK-activation loop, and the N-terminal domain inhibits JAK Tyr kinase activity, thus preventing further activation of STAT-1 (4–6). A role for the SOCS box was suggested by analogy with the Von Hippel–Lindau tumor-suppressor protein where interaction with elongins B/C targets associated substrates (the HIF-1α transcription factor) to proteasomal degradation (17). Paradoxically, however, data have been presented that loss of the SOCS box has no effect on the ability of SOCS-1 to inhibit STAT activation in over-expression systems *in vitro* (4–6), and that instead, it leads to instability of the SOCS-1 protein itself (5, 14). An explanation of these observations is offered by the present results. In over-expression systems, direct inhibition of the JAK/STAT pathway by the SH2 and N-terminal domains is sufficient to completely inhibit this pathway so no further effect is seen through the SOCS box. However, at physiological SOCS-1 levels, only partial inhibition of the pathway may be achieved by the former mechanism, and additional inhibition then is achieved by targeting bound proteins to proteasomal destruction through the SOCS box.

The selective loss of truncated SOCS-1 in heterozygous (Δ/+) animals (Figs. 2*c* and 4*c*) confirms the previously reported relative instability of the truncated compared with the full-length protein in cell lines (5, 14) and suggests that the SOCS box, by recruiting other proteins to the complex, also may have a role in stabilizing SOCS-1. This finding is consistent with the recent observation that binding of the elongin B/C complex stabilizes the von Hippel–Lindau protein against proteasomal degradation (23). The elevated levels of the truncated protein in Δ/Δ compared with Δ/+ mice could be a result not only of biallelic expression but also of the sustained elevation of STAT-1 (which induces SOCS-1 expression; ref. 3) or the inability of the truncated proteins to recruit proteasomal complexes that destroy associated signaling molecules as well as SOCS-1 in Δ/Δ mice. In either case, the present study indicates clearly that the SOCS box is an important element in the SOCS-1 protein that contributes to inhibition of cytokine signaling. It also suggests that the independent ability of this domain to recruit E3 ubiquitin ligase activity will provide an important clue to the roles of the many other proteins that contain a SOCS box.

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Ankyrin Repeat and SOCS Box Containing Protein Asb-9 Targets Creatine Kinase B For Degradation

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SUMMARY

The suppressors of cytokine signalling (SOCS) proteins inhibit cytokine signalling by direct interaction with Janus kinases (JAKs) or activated cytokine receptors. In addition to the N-terminal and SH2 domains that mediate these interactions, SOCS proteins contain a C-terminal SOCS box. DNA database searches have identified a number of other protein families that possess a SOCS box of which the ankyrin repeat and SOCS box-containing (Asb) proteins constitute the largest. While it is known that the SOCS proteins are involved in the negative regulation of cytokine signalling, the biological and biochemical functions of the Asbs are largely undefined. To understand the functional role of Asb proteins, a proteomic approach was implemented and creatine kinase B (CKB) was shown to interact with Asb-9 in a specific, SOCS box independent manner. Furthermore, transfection of increasing concentrations of a FLAG-tagged Asb-9 construct into 293T cells increased polyubiquitination of CKB and resulted in a concomitant decrease in total CKB levels within the cell. The targeting of CKB for degradation by Asb-9 was entirely SOCS box dependent and suggests that Asb-9 may act as a specific ubiquitin ligase regulating CKB abundance.

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INTRODUCTION

The suppressor of cytokine signalling (SOCS) proteins function as part of a classical negative feedback loop, attenuating cytokine action through inhibition of the JAK/STAT signal transduction pathway (1). The SOCS proteins are structurally characterized by an N-terminal region, a central SH2 domain and a conserved C-terminal motif of approximately 40 amino acids, termed the SOCS box. Structural and functional analyses have shown that SOCS proteins directly interact with activated JAKs and cytokine receptors through their N-terminal and SH2 domains to mediate their effects (2). However, recent *in vivo* evidence revealed that for a complete termination of signal transduction, the SOCS box is also required (3).

The SOCS box was first identified in the SOCS proteins and has since been found in more than 50 proteins across a range of species (4,5). These proteins have been sub-divided into nine different families based on the type of domain or motif they possess upstream of the SOCS box and include eighteen ankyrin repeat-containing SOCS box proteins (ASBs), four SPRY-domain proteins with a SOCS box (SSBs), two WD40-repeat proteins with a SOCS box (WSBs), the tubby-domain-containing proteins with a SOCS box, a family of small GTPases and the Neuralized family of proteins (4,5). It has been shown that the SOCS box from several of these family members binds elongin C, which in turn associates with a complex consisting of elongin B, a cullin family member (Cullin-2 or Cullin-5) and a RING-finger protein called Roc1 or Rbx-1 (5-7). This protein complex constitutes an E3 ubiquitin ligase termed the ECS (elongin C-cullin-SOCS box) that, together with a ubiquitin activating enzyme (E1) and a ubiquitin-

conjugating enzyme (E2), facilitates the polyubiquitination of proteins bound to the SH2 domain of SOCS family members. It has been suggested by several groups that the SOCS box functions as an adaptor, linking specific protein-protein interaction domains to generic components of the ubiquitin ligase machinery. This results in the polyubiquitination and proteasomal degradation of bound proteins thereby regulating protein levels within the cell (8,9). It is interesting to note that other studies suggest an additional role for the SOCS box, in particular that the SOCS box-elongin B/C interaction may act to stabilize SOCS proteins, thereby protecting SOCS proteins from degradation (10-12).

The Asbs constitute the largest family of SOCS box-containing proteins, with eighteen murine and human Asbs identified, yet their biological and biochemical functions remain to be clearly defined. The Asbs contain a protein interaction motif upstream from the SOCS box composed of a variable number of ankyrin repeats. The ankyrin repeat consensus sequence is 33 amino acids in length and can be found in many eukaryotic, bacterial and viral proteins with various functions including receptors, cell cycle regulators, secreted proteins, tumor suppressors and transcription factors (reviewed in (13). Each ankyrin repeat comprises a V-shaped helix-turn-helix motif, linked together by loops. The repeats are stacked consecutively in bundles providing a stable platform for protein-protein interactions (reviewed in (14)).

Recent publications have implicated the Asbs in a number of different processes. For example, Asb-5 may play a role in the initiation of arteriogenesis (15) while Asb-15 may be involved in muscle growth (16). Two separate studies suggest that Asb-2 may regulate myeloid cell

proliferation and/or differentiation. It is thought that genes critical to the maturation of normal hematopoietic progenitors are repressed in cells undergoing leukemic transformation and that these genes can be induced in leukemic cells by retinoic acid (RA). Both groups reported the rapid induction of Asb-2 by RA in leukemic cells, via activation of a retinoic acid receptor-binding element present in the Asb-2 gene promoter. Furthermore, in promyelocytic leukemia cell lines, Asb-2 expression led to growth arrest and chromatin condensation, events which are characteristic of hematopoietic cell commitment to terminal differentiation (17,18). Interestingly, Asb-8 has also been implicated in cancer. Liu *et al.* found that while Asb-8 was not expressed in normal adult lung tissue, the transcript was detected in several lung carcinoma cell lines. Furthermore, transfection of a possible dominant negative form of Asb-8 (human Asb-8 cDNA lacking the SOCS box), suppressed the growth of lung adenocarcinoma cells *in vitro*, hinting at an association of Asb-8 with the development of lung cancer (19).

Creatine kinase is critical for the maintenance and regulation of cellular energy stores in tissues with high and rapidly-changing energy demands such as skeletal and cardiac muscle and the brain. In mammals, three cytosolic dimeric isoforms of creatine kinase are expressed: muscle-type (CKM), brain-type (CKB) and a heterodimer of both muscle and brain subunits (CKMB) that is predominantly expressed in the heart. In addition, two mitochodrial CK isoforms have been identified and characterized. The brain-type cytosolic enzyme of creatine kinase, CKB, plays a major role in cellular energy metabolism of non-muscle cells. It is expressed in a range of tissues, mainly in the brain and retina, but also in uterus, placenta, kidney and testes. There is ample evidence that the CK system is linked with brain and muscle function (reviewed in (20).

Ankyrin Repeat and SOCS Box Containing Protein Asb-9 Targets Creatine Kinase B For Degradation

A number of neurological and muscular diseases display perturbations in CK activity and creatine metabolism although the causal relationships of many are not known. A role for CKB in brain function is further supported by altered behavioral patterns observed in CKB knockout mice (21).

Creatine kinase has also been connected to malignancy and cancer. The over-expression of CKB has been observed in a number of tumors including neuroblastoma, small-cell lung carcinoma, colon and rectal adenocarcinoma and breast and prostate carcinoma as well as some tumor cell lines (reviewed in (22) and (20)). Elevated CKB gene expression was also reported in B-lineage cells from patients with acute lymphoblastic leukemia (23). Furthermore, the gene encoding CKB is subject to regulation by both an oncogene and a tumor suppressor gene. A series of tissue culture co-transfection and infection experiments demonstrated that the adenovirus E1a oncogene induced both CKB enzymatic activity and mRNA levels (24). Findings from transient transfection experiments with wild-type or mutant p53 in HeLa cells (human cervical carcinoma cells transformed by human papilloma virus) showed that wild-type p53 repressed the CKB promoter (25). It is interesting to note that many human small cell lung carcinomas, which exhibit elevated CKB expression, contain mutations in p53 alleles (reviewed in (20)).

Given the evidence that SOCS box-containing proteins target specific proteins for degradation via the E3 ubiquitin ligase complex recruited by the SOCS box, we reasoned that the key to elucidating the function of the Asb protein family is to study the proteins with which they interact. Here, we report the identification of CKB as a specific binding protein of Asb-9 and

show that this interaction leads to CKB ubiquitination and degradation in a SOCS box-dependent manner.

EXPERIMENTAL PROCEDURES

Northern Hybridization- Tissues were dissected from 8 week old C57B/6 mice and immediately snap frozen in liquid N_2. Total RNA was extracted from tissues using Trizol reagent according to the manufacturer's instructions (Invitrogen). Northern blots were performed after electrophoresis as described (26). For Northern blot hybridization, the entire coding region of the mouse Asb-9 cDNA was used. The membrane was stripped and re-hybridized with a 1.2 kbp *PstI* fragment of the chicken glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA to control for RNA loading and integrity.

Expression Vectors- The cDNAs encoding Asbs-1-6,-8 ,-9 and -11, SOCS-3, SSB-1 and SSB-2, WSB-1 and WSB-2 were obtained as described (2,4,27). Constructs encoding these proteins, with or without the SOCS box, with an N-terminal FLAG epitope tag (DYKDDDDK) were generated by PCR to give fragments with in-frame *MluI* restriction enzyme sites at both the N- and C-termini and were subcloned into the mammalian expression vector pEF-FLAG-I.

Transfection of 293T Cells with Asb-9- Human embryonic kidney 293T cells were plated at a density of 8×10^6 cells per Nunclon T175 cm^2 tissue culture flask (Nalge Nunc International) or in 6 well Costar plates (Corning Incorporated) at 0.5×10^6 cells/well and cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% [v/v] fetal calf serum (HyClone Laboratories). Cells were incubated overnight at 37°C in an humidified atmosphere of 10% CO_2 in air and transfected with 2.5 μg of pEF-FLAG-I expression vector containing the cDNA of

interest, using FuGene transfection reagent (Roche) according to the manufacturer's instructions. Where indicated, the proteasomal inhibitor PS341 (a gift from Dr. John Silke, The Walter and Eliza Hall Institute of Medical Research) was used at a concentration of 10 nM diluted in DMSO. Cells were treated with PS341 for 24 hours.

Cell Lysis and Affinity Purification- Cells were lysed in NP-40 buffer (0.5% [v/v] Nonidet P-40, 10 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 10% glycerol) containing protease inhibitors (Complete cocktail tablets, Roche) for 30 minutes on ice. For the ubiquitination studies, cells were treated with KALB lysis buffer (150 mM NaCl, 50 mM Tris [pH 7.5], 1% [v/v] Triton X-100, 1 mM EDTA, 1 mM Na_3VO_4, 1 mM NaF) containing protease inhibitors (Complete cocktail tablets, Roche). For large-scale affinity purification, clarified lysates were incubated with anti-FLAG M2 resin for 3-4 hours at 4°C and then poured into Poly-prep chromatography columns (BioRad) to recover the M2 beads. The affinity resin was then subjected to 5 x 2 mL washes with lysis buffer. Bound proteins were subsequently eluted with 8 x 0.2 mL of 200 µg/mL FLAG peptide. Eluates were pooled and concentrated to 40 µL using a Millipore concentration unit (molecular weight cut-off of 10,000), mixed with 15 µL of 4x SDS sample buffer containing 0.2 M DTT and resolved on a 4-20% gradient gel (Novex). The gel was stained with 0.1% Coomassie Blue (Pierce) in 50% [v/v] methanol and destained in 12% [v/v] methanol and 7% [v/v] acetic acid.

Protein Identification by Tryptic Digest and Mass Spectrometry- Protein bands were excised and digested *in situ* using trypsin (28) . Peptides were separated by capillary chromatography (29)

and sequenced using an on-line electrospray ionization ion-trap mass spectrometer (ESI-IT-MS) (LCQ Thermo-Finnigan, San Jose, CA, USA). Operating conditions for ESI-IT-MS and MS data analysis are described elsewhere (30). Automatically selected tryptic peptide ions were identified using the SEQUEST algorithm incorporated into the Finnigan Xcalibur™ software (31). A non-redundant protein database produced by the Office of Information Technology of the Ludwig Institute for Cancer Research was used.

Western Blot Analysis- Proteins were resolved by SDS-PAGE, transferred to PVDF-Plus membranes and blocked for 1 hour in 5% [w/v] skim milk powder. Primary antibody was diluted in blocking solution and incubated with the membrane for one hour. FLAG-tagged proteins were detected by rat anti-FLAG antibody (9H1)(32), while endogenous CKB was detected by antibody raised against a peptide mapping at the amino terminus of CKB (sc-15157, Santa-Cruz). Antibody binding was visualized using appropriate HRP-conjugated secondary antibodies and the enhanced chemiluminescence (ECL) system (Amersham) according to the manufacturer's instructions.

Generation of MYC tagged CKB and Detection of Ubiquitinated Protein- A cDNA clone encoding creatine kinase B in a pCMV-SPORT6 vector was purchased from the I.M.A.G.E Consortium (supplied by the MRC GeneService) (ID 4225384). Oligonucleotides (5'-ACGTGGCGCGCCAGCCCTTCTCCAACAGCCATAATACG-3' and (5'-ACGTACGCGTCTGGGCCGGCATGAGGTCATC-3') were used to amplify the CKB coding sequence with in frame *AscI* and *MluI* sites at the 5' and 3' ends. The PCR-generated fragment

was digested with *AscI* and *MluI* then subcloned into pEF-MYC-I to generate a CKB construct with a C-terminal MYC (DQKLISEEDL) tag. The MYC-tagged CKB plasmid, a HA-tagged ubiquitin plasmid (a generous gift from Dr. John Silke, The Walter and Eliza Hall Institute of Medical Research) and a FLAG-tagged Asb-9 plasmid or its deleted SOCS box form were transfected into 293T cells as described above. Clarified cell extracts were immunoprecipitated with anti-MYC antibody and ubiquitinated protein detected by anti-HA antibody (Roche).

Pulse Chase Analysis of Creatine Kinase B – 293T cells were transfected with the MYC-tagged CKB plasmid and either the pEFBOS expression vector, FLAG-Asb-9 or FLAG-Asb-9/ΔSB plasmids as described above. At 48 hours post-transfection, cells were rinsed with methionine-free DMEM supplemented with 0.1% BSA (AlbuMAX I 10% solution, GibcoBRL). Cells were radiolabeled for 1 hour with 0.1 mCi [^{35}S] methionine–cysteine mixture (NEG-072, Perkin-Elmer) per mL of methionine-free culture medium. Cells were then rinsed to remove pulse-labeling medium and chased in normal culture medium. Cell lysates were immunoprecipitated with anti-MYC antibody as described. Protein was eluted with 40 µL of SDS loading buffer, separated by SDS-PAGE and transferred onto PVDF-Plus membranes. The [^{35}S]-labeled MYC-tagged CKB protein was detected using a PhosphorImager (Molecular Dynamics) and quantified by densitometry using ImageQuant software (version 5.0). The fraction of [^{35}S]-labeled MYC-CKB remaining at each time point was then calculated to allow the half-life of the protein to be estimated.

Ankyrin Repeat and SOCS Box Containing Protein Asb-9 Targets Creatine Kinase B For Degradation

RESULTS

Expression of Asb-9 in vivo. In the adult mouse, Asb-9 mRNA expression was detected predominantly in the testes and kidney, with low expression observed in the heart and liver (Figure 1). Asb-9 expression was undetectable in all other tissues examined. Given the expression of Asb-9 mRNA in kidney, the human embryonic kidney 293T cell line was utilized for subsequent experiments.

Identification of CKB as an Asb-9 Specific Interacting Protein. To identify proteins that associate with Asb-9, anti-FLAG M2 affinity resin was used to purify proteins from lysates derived from 293T cells expressing FLAG-tagged Asb-9. The M2-immunoprecipitates were separated by SDS polyacrylamide gel electrophoresis (Figure 2) and proteins of various molecular weights were observed to co-immunoprecipitate with the Asb-9 protein (lane 2). A number of these were not seen in immunoprecipitates from the control 293T cells (lane 1). The proteins were excised from the gel, digested with trypsin *in situ* and identified by mass spectrometry (Table 1). The Hsp60/70 proteins have been observed to co-immunoprecipitate with a plethora of other proteins and are likely to be non-specific (Benjamin T. Kile, Jian-Guo Zhang, Nicos A. Nicola; unpublished observations). Also, consistent with experiments with other SOCS box-containing proteins, elongins B and C (18 kDa and 15 kDa, respectively) and cullin 5 (90 kDa) co-immunoprecipitated with Asb-9. In contrast, creatine kinase B had not been previously identified in several SOCS protein immunoprecipitation experiments and thus appeared to be interacting in a specific manner and worthy of further analysis.

Ankyrin Repeat and SOCS Box Containing Protein Asb-9 Targets Creatine Kinase B For Degradation

Specificity of Asb-9-CKB Interaction. The specificity of the Asb-9-CKB interaction was examined by determining whether CKB could interact with other SOCS box-containing proteins. Various FLAG-tagged SOCS box-containing proteins were expressed in 293T cells, including full length and/or SOCS box deleted forms of Asbs-1-6,-8 ,-9 and -11, SOCS-3, SSB-1 and SSB-2, WSB-1 and WSB-2. The FLAG-tagged proteins were immunoprecipitated from clarified cell lysates, separated by SDS PAGE, and association with CKB was detected by western blot with anti-CKB antibody. Results showed that CKB was only detected in lysates of cells transfected with Asb-9 suggesting that, at least in the panel of SOCS box proteins tested, CKB interacts specifically with Asb-9 (Figure 3A and 3B).

SOCS box dependent and independent interactions. In order to study the basis of the Asb-9 and CKB interaction further, full-length FLAG-Asb-9 or Asb-9 lacking the SOCS box (Asb-9/ΔSB) was transiently expressed in 293T cells and the interaction with CKB examined. As shown in Figure 4A, both Asb-9 and Asb-9/ΔSB readily interacted with endogenous CKB. This suggested that the binding of CKB to Asb-9 occurs independently of the SOCS box. In contrast, the SOCS box was critical for interactions with elongins B and C (Figure 4C) and Cullin 5 (Figure 4D) as has been shown for several other proteins (Figure 2) (6,10).

Asb-9 Targets CKB For Degradation in a SOCS Box Dependent Manner. To examine the consequences of the interaction between Asb-9 and CKB, 293T cells were co-transfected with increasing concentrations (0-2.5 μg) of FLAG-tagged Asb-9 or FLAG-tagged Asb-9/ΔSB constructs and decreasing concentrations of pEF-FLAG vector. This was to ensure that a total of

2.5 μg of DNA was used per transfection. Total cell lysates were analyzed by Western blot with anti-CKB antibody and it is notable that as the concentration of transfected Asb-9 increased (Figure 5A), endogenous CKB levels decreased, suggesting that Asb-9 may play a role in regulating the levels of CKB within the cell. In contrast, no decrease of CKB levels was observed in cells expressing Asb-9/ΔSB (Figure 5B), indicating that, while the binding of CKB by Asb-9 occurs via the ankyrin repeat, the effect on protein levels is dependent on an intact SOCS box.

To assess further the effect of Asb-9 on CKB degradation, the turnover of the CKB protein was determined via pulse-chase analysis. Due to the absence of an effective CKB antibody for immunoprecipitation, 293T cells were co-transfected with a MYC-tagged CKB plasmid and either a pEFBOS vector control, FLAG-tagged Asb-9 or FLAG-tagged Asb-9/ΔSB plasmids. Transfected cells were pulse-labeled with [^{35}S]-methionine and then chased for various time periods in normal culture medium containing unlabeled methionine (Figure 6). MYC-tagged CKB was immunoprecipitated with anti-MYC antibody and radioactive bands were visualized using a PhosphorImager. This analysis showed that the turnover of CKB was accelerated by co-expression of Asb-9 and that the SOCS box is critical for this effect as CKB half-life in the presence of Asb-9/ΔSB followed a similar degradation over time as that seen with the vector control samples (Figure 6A and 6B). Finally, treatment with the proteasomal inhibitor PS341 prolonged CKB half-life (Figure 6B). These results indicate that Asb-9 promotes the degradation of CKB and that the degradation is SOCS box dependent and is mediated by the proteasome.

Ankyrin Repeat and SOCS Box Containing Protein Asb-9 Targets Creatine Kinase B For Degradation

Asb-9 Induces SOCS Box-Dependent Ubiquitination of CKB: SOCS proteins have been reported to induce proteasome-dependent degradation of their target proteins (33,34). To investigate whether Asb-9-induced reduction of CKB levels was due to SOCS box-mediated ubiquitination of CKB, we co-expressed MYC-CKB with full-length Asb-9 or Asb-9/ΔSB as well as HA-ubiquitin. As shown in Figure 7A, little polyubiquitination of CKB was observed in the absence of co-expression of wild-type Asb-9 (lanes 2 and 3); however, co-expression of wild-type Asb-9 resulted in enhanced polyubiquitination of immunoprecipitated CKB (lane 4). This was not apparent in cells transfected with Asb-9 lacking the SOCS box (lane 5). Upon treatment of cultures with the proteasomal inhibitor PS341, an increased level of ubiquitinated CKB was observed (Figure 7A - lanes 6, 7 and 8), demonstrating that PS341 effectively inhibits the degradation of ubiquitinated proteins by inactivating the proteasome. Importantly, the ubiquitination of CKB was substantially enhanced on co-expression with Asb-9 (lane 7), supporting our previous observations. To confirm that the ubiquitinated protein smears observed in the Asb-9 co-transfections (lanes 4 and 7) are due to modification of the MYC-tagged CKB protein by ubiquitination, the blot was stripped and re-probed with anti-MYC (panel B). The re-probe shows a strong band that most likely corresponds to a mono-ubiquitinated form of CKB, and was predominant when Asb-9 was co-expressed. As expected, the band appeared more intense following treatment with PS341. Unmodified MYC-CKB protein was easily detected and no obvious differences in levels were observed in the untreated versus PS341 treated samples. This may be attributed to the immunoprecipitation process, as an increase in MYC-

tagged CKB protein levels upon PS341 treatment was observed when total cell lysate was examined via immunoblot with anti-MYC (panel D).

DISCUSSION

To elucidate the function of the Asb protein family, it is important to study the proteins with which they interact. By using a proteomic approach, our studies identified creatine kinase B (CKB) as a protein capable of specifically interacting with Asb-9. CKB is a key cytosolic enzyme in cell energy metabolism (reviewed in (35)). It reversibly catalyses the ATP-dependent phosphorylation of creatine and hence, provides an ATP buffering system for tissues requiring large amounts of energy.

Asb-9 is one of eighteen members of the ankyrin repeat-containing SOCS box protein family (Asbs). Although this family represents the largest family of all SOCS box-containing proteins, their biological and biochemical functions remain poorly defined. Ankyrin repeats are a structural motif involved in protein-protein interactions (reviewed in (14)) whereas the SOCS box interacts specifically with elongin C. SOCS box-containing proteins have been suggested to act as part of an E3 ubiquitin ligase complex with the specificity of the complex determined by the protein interaction motif located upstream from the SOCS box (reviewed in (5)).

Our results demonstrate that the specific interaction between Asb-9 and CKB occurs independently of the SOCS box and that the interaction with CKB leads to a SOCS box-dependent polyubiquitination of CKB, resulting in a subsequent decline in cellular CKB levels. It would be interesting to examine this interaction in primary cells and *in vivo* when antibodies become available. In this paper, we reported that Asb-9 was expressed in the testes and kidneys with low expression in heart and liver. CKB expression overlaps with that of Asb-9 in some tissues. However, CKB is predominantly expressed in the brain. The Asb-9 transcript was not detected in brain in our studies, although expression has been reported in the hypothalamus (GenBank Accession No. BB173163.1). A detailed analysis of CKB and Asb-9 expression levels in various tissues, including different regions of the brain, would therefore be of value.

In addition, CKB is over-expressed in a wide range of solid tumors and tumor cell lines and has been used as a prognostic marker of cancer and metastasis, although this application remains controversial (20). Furthermore, the gene encoding CKB is subject to positive regulation by the oncogene E1a and negative regulation by the tumor suppressor gene, p53 (22,36). Also, many growth factors and hormones such as estrogen stimulate CKB (37,38). Estrogen has been shown to highly induce expression of creatine kinase B in the female rat reproductive tract, as well as in human breast tumors and tissues (39). It is not known which factors if any induce the expression of Asb-9 but it is possible that regulators of CKB might also exert effects on Asb-9 activity and expression. It has been proposed that the CK system is involved in tumor growth through regulation of ATP production or modulation of as yet undefined processes. Molecules that disrupt this system may have an impact on tumor growth or progression. Given the interaction

of Asb-9 with CKB and implied roles of Asb-2 and Asb-8 in cancer, it is tempting to speculate that Asb-9 may also have a role in tumor development but this will require further study.

It is clear that the mechanism of the Asb-9-CKB interaction needs to be further examined. Preliminary results presented in this paper show that Asb-9 may function in a similar way to SH2-containing SOCS proteins since some of the key players that are involved in the SOCS-mediated protein degradation pathway are also present in the Asb-9-CKB interaction, specifically cullin-5 and elongins B and C. The SOCS proteins target key signaling proteins, such as the JAKs and receptors, for degradation by the proteasome, thereby attenuating cytokine and tyrosine kinase receptor signaling. While the biological setting of the Asb-9-CKB interaction is not known, we propose that Asb-9 targets CKB for proteasomal degradation in a similar manner.

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Ankyrin Repeat and SOCS Box Containing Protein Asb-9 Targets Creatine Kinase B For Degradation

FIGURE LEGENDS

Figure 1: Northern blot hybridization analysis of Asb RNA expression levels. Total RNA was isolated from primary tissues taken from 8 week old normal mice. The transcript size for Asb-9 is 1.3 kb. To confirm RNA loading and integrity, the blot was stripped and re-probed with GAPDH.

Figure 2: Identification of Proteins that Bind to Asb-9. Lysate from 293T cells transfected with pEFBOS (lane 1) and PEF-FLAG-tagged Asb-9 (lane 2) were subjected to precipitation with anti-FLAG M2 resin. Bound proteins were separated by SDS-PAGE and visualized by Coomassie staining. Arrows in lane 2 indicate the protein bands excised for sequencing analysis by mass spectrometry (see Table 1).

Figure 3: CKB specifically interacts with Asb-9. In panels A and B membranes were immunoblotted with anti-CKB antibody. Arrows indicate the CKB band. Expression of all FLAG-tagged proteins were confirmed by re-probing with anti-FLAG antibody as shown in panels C and D. /ΔSB represents SOCS box deleted constructs.

Figure 4: SOCS box dependent and independent interactions. Transfected 293T lysates were immunoprecipitated with M2 resin and immunoblotted with anti-CKB, anti-elongin B/C or anti-Cullin 5 (panels A, C and D respectively). Expression of transfected protein was confirmed by an anti-FLAG Western (panel B).

Figure 5: SOCS box dependent degradation of CKB. 293T cells were co-transfected with increasing concentrations (0-2.5 μg) of FLAG-tagged Asb-9 or FLAG-tagged Asb-9/ΔSB constructs and decreasing concentrations of pEFBOS vector to ensure that a total of 2.5 μg of DNA was used per transfection. Total cell lysate was immunoblotted with anti-CKB (panels A and B). Expression of FLAG-tagged protein was determined by an anti-FLAG western blot (panels C and D). Actin levels were also examined in the presence of increasing concentrations of Asb-9 to ensure that Asb-9 specifically regulates CKB degradation (panels E and F).

Figure 6: Degradation of CKB is enhanced by Asb-9 co-expression. (A) Asb-9 co-expression enhances the degradation of CKB in vitro. 293T cells were transfected with MYC-tagged CKB (0.5 μg) and either pEFBOS vector, FLAG-tagged Asb-9 or FLAG-tagged Asb-9/ΔSB plasmids (0.5 μg). At 48 hours, cells were pulsed for 1 hour with [^{35}S]-methionine labeling mixture then chased for 0, 0.5, 1, 2, 4, 8 and 24 hours in DMEM with 10% FCS. Where indicated, cultures were treated prior to lysis with 10 nM PS341 overnight. Lysates were subjected to immunoprecipitation with anti-MYC antibody and bands visualized by a PhosphorImager. Images are representative of at least 3 independent experiments giving similar results. (B) The extent of [^{35}S]-methionine labeled CKB radioactivity was quantified by densitometry. As shown, the addition of PS341 prolongs CKB half-life when co-expressed with Asb-9. Graph constructed from data obtained from at least 3 separate pulse-chase experiments.

Ankyrin Repeat and SOCS Box Containing Protein Asb-9 Targets Creatine Kinase B For Degradation

Figure 7: Asb-9 Induces SOCS Box-Dependent Ubiquitination of CKB. 293T cells were untransfected (lane 1), transfected with HA-ubiquitin (1.0 μg) (lane 2), transfected with HA-ubiquitin (0.5 μg) and MYC-CKB (0.5 μg) (lanes 3 and 6), transfected with HA-ubiquitin (0.5 μg), MYC-CKB (0.5 μg) and FLAG-Asb-9 (0.5 μg) (lanes 4 and 7) or FLAG-Asb-9/ΔSB (0.5 μg) (lanes 5 and 8). Where indicated, cultures were treated prior to lysis with 10 nM PS341 overnight. 48 hours post transfection, cells were lysed in NP40 lysis buffer. Cell lysates were subjected to anti-MYC immunoprecipitation, followed by immunoblotting with anti-HA antibody (panel 7A). The blot was then stripped and re-probed with anti-MYC antibody to show ubiquitinated forms of MYC-tagged CKB protein. Mono-ubiquitinated CKB forms were determined by calculating R_f values (calculations not shown) (panel 7B). Total cell lysates were subjected to immunoblot with anti-FLAG antibody to confirm expression of transfected FLAG-tagged proteins and anti-MYC to confirm expression of MYC-tagged CKB protein (panels 7C and 7D respectively).

Table 1

Table 1: Mass Spectrometric Identification of Proteins

Protein(s) Identified	Database/Accession no.	Mol wt (kDa)	No. of peptides identified	Sequence Coverage (%)
Asb-9	TrEMBL, Q91ZT8	31.6	17	76.6
Creatine kinase B	SwissProt, P12277	42.6	31	73.2
60 kDa heat shock protein	SwissProt, P10809	61.0	32	77.0
70 kDa heat shock protein*	SwissProt, P08107	70.0	29	61.8
Cullin 5*	SwissProt, O93034	90.8	23	35.5
Elongin B*	TrEMBL, Q15370	13.1	17	93.2
Elongin C*	TrEMBL, Q15369	12.4	4	56.3

* Asterisk indicates proteins identified in separate purification experiments

Figure 1

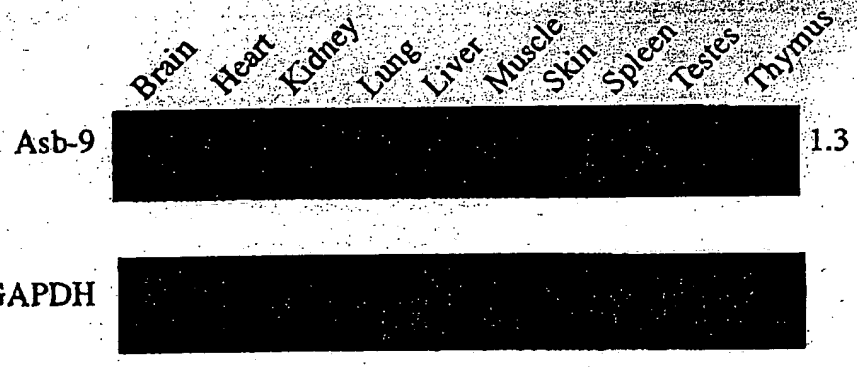

Brain
Heart
Kidney
Lung
Liver
Muscle
Skin
Spleen
Testes
Thymus
Asb-9
1.3
GAPDH

Figure 2

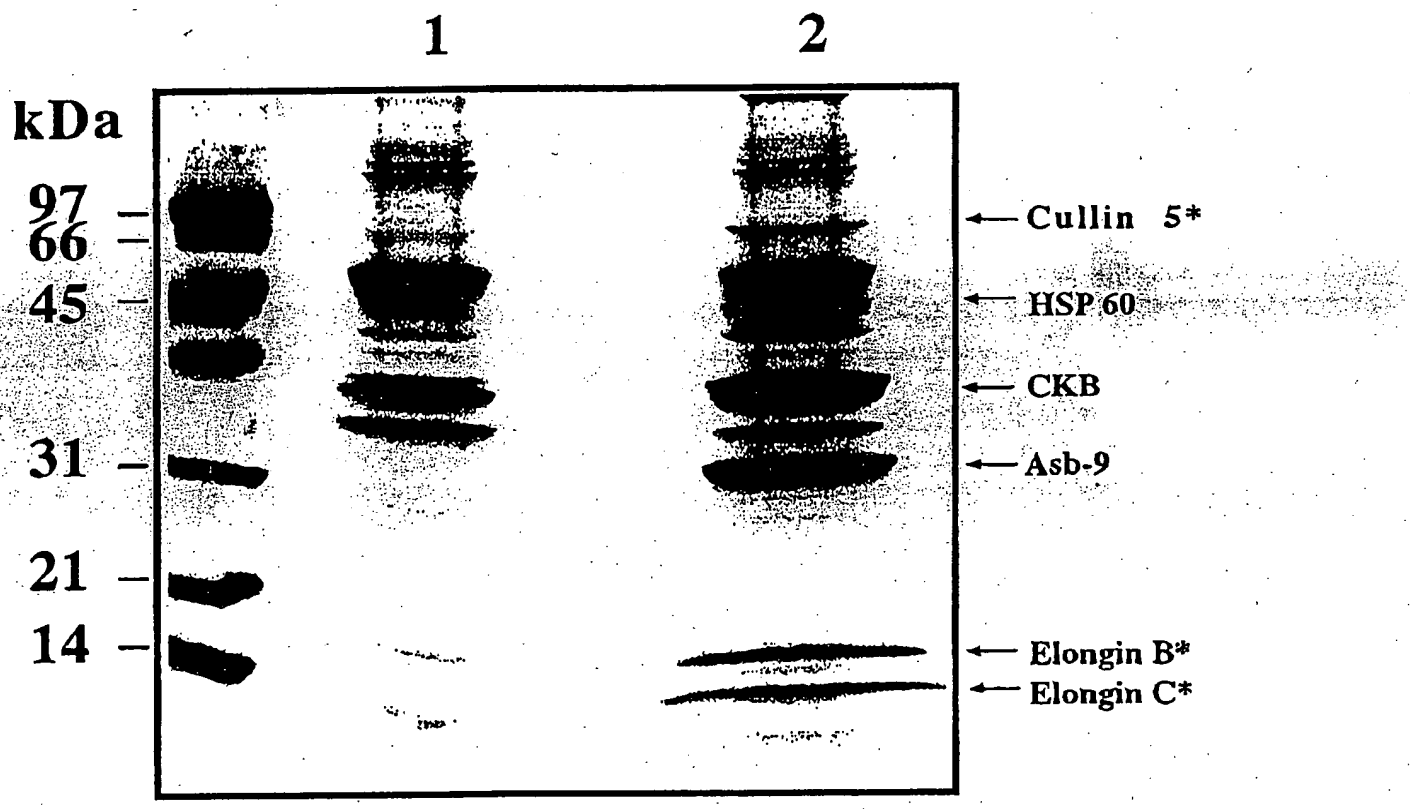
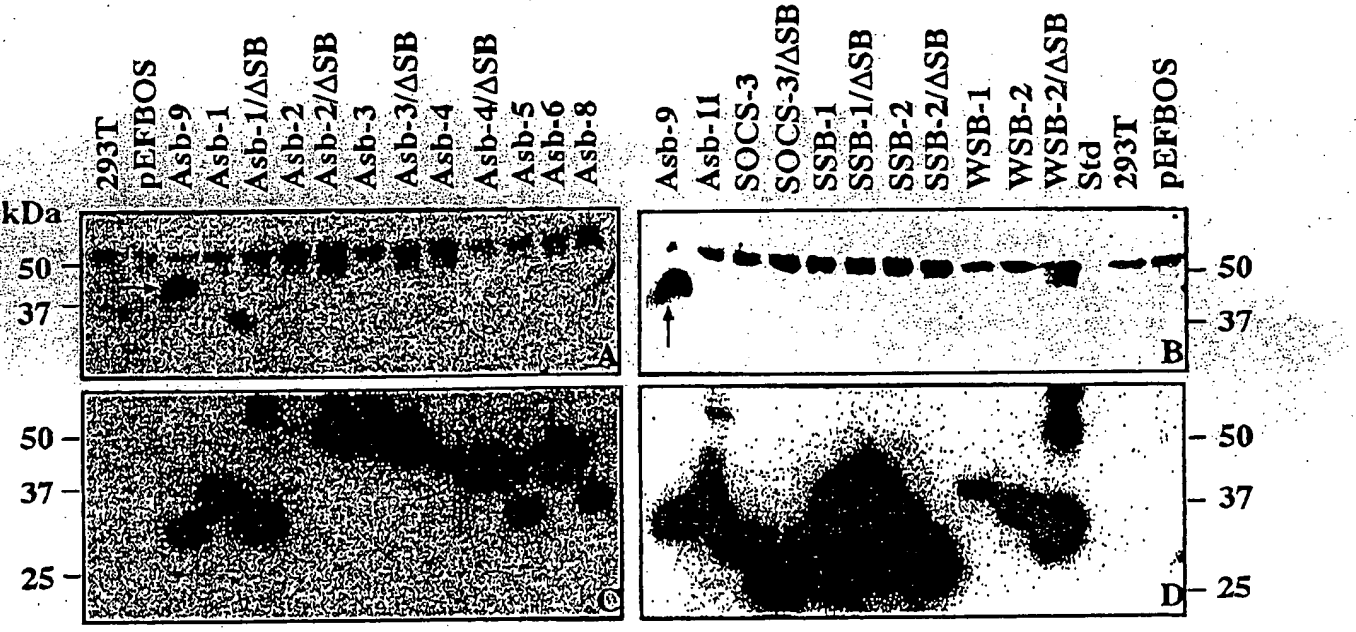

1
2
kDa
97
66
45
31
21
14
Cullin 5*
HSP 60
CKB
Asb-9
Elongin B*
Elongin C*

Figure 3


293T
pEFBOS
Asb-9
Asb-1
Asb-1/ΔSB
Asb-2
Asb-2/ΔSB
Asb-3
Asb-3/ΔSB
Asb-4
Asb-4/ΔSB
Asb-5
Asb-6
Asb-8
kDa
50
37
A
Asb-9
Asb-11
SOCS-3
SOCS-3/ΔSB
SSB-1
SSB-1/ΔSB
SSB-2
SSB-2/ΔSB
WSB-1
WSB-2
WSB-2/ΔSB
Std
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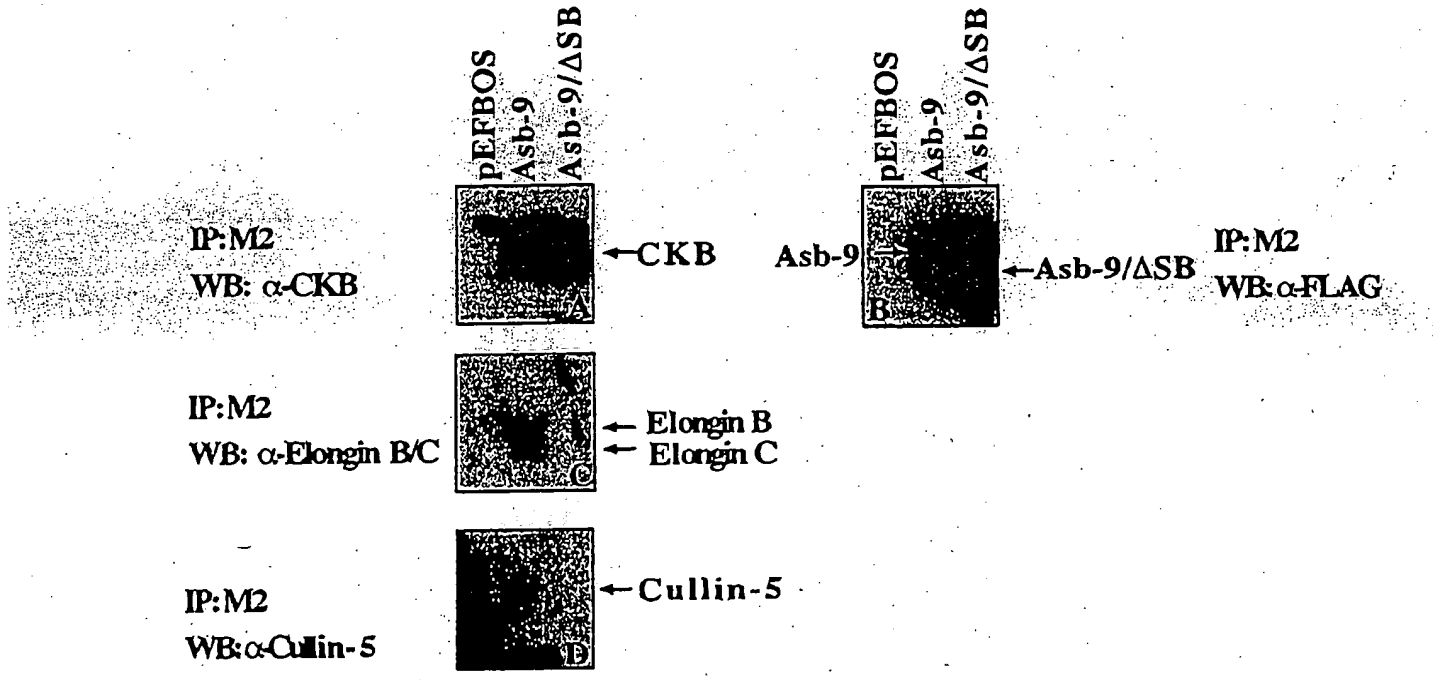
pEFBOS
50
37
B
50
37
25
C
50
37
25
D

Figure 4


pEFBOS
Asb-9
Asb-9/ΔSB
IP:M2
WB: α-CKB
CKB
A
pEFBOS
Asb-9
Asb-9/ΔSB
Asb-9
Asb-9/ΔSB
IP:M2
WB:α-FLAG
B
IP:M2
WB: α-Elongin B/C
Elongin B
Elongin C
C
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Cullin-5
D

Figure 5

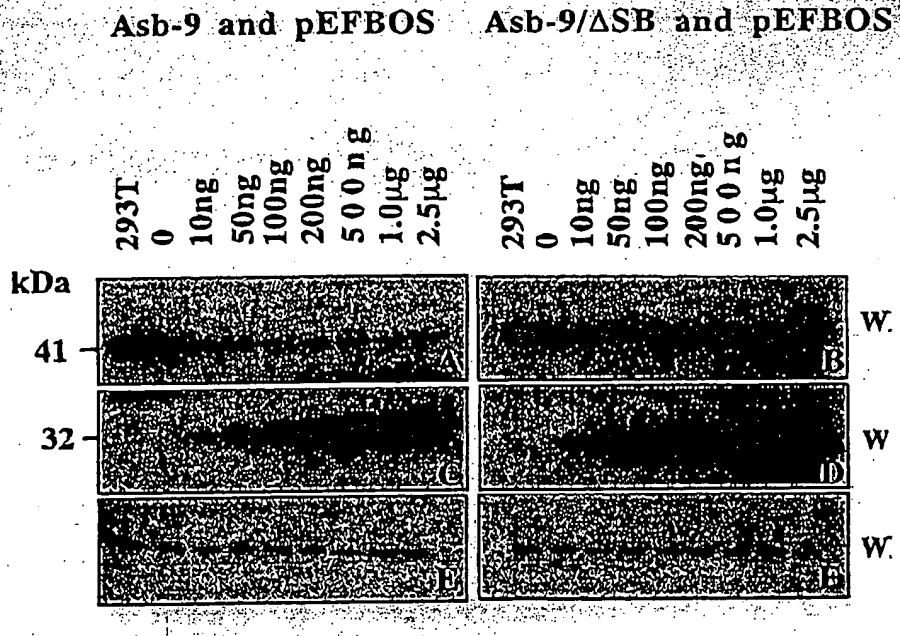
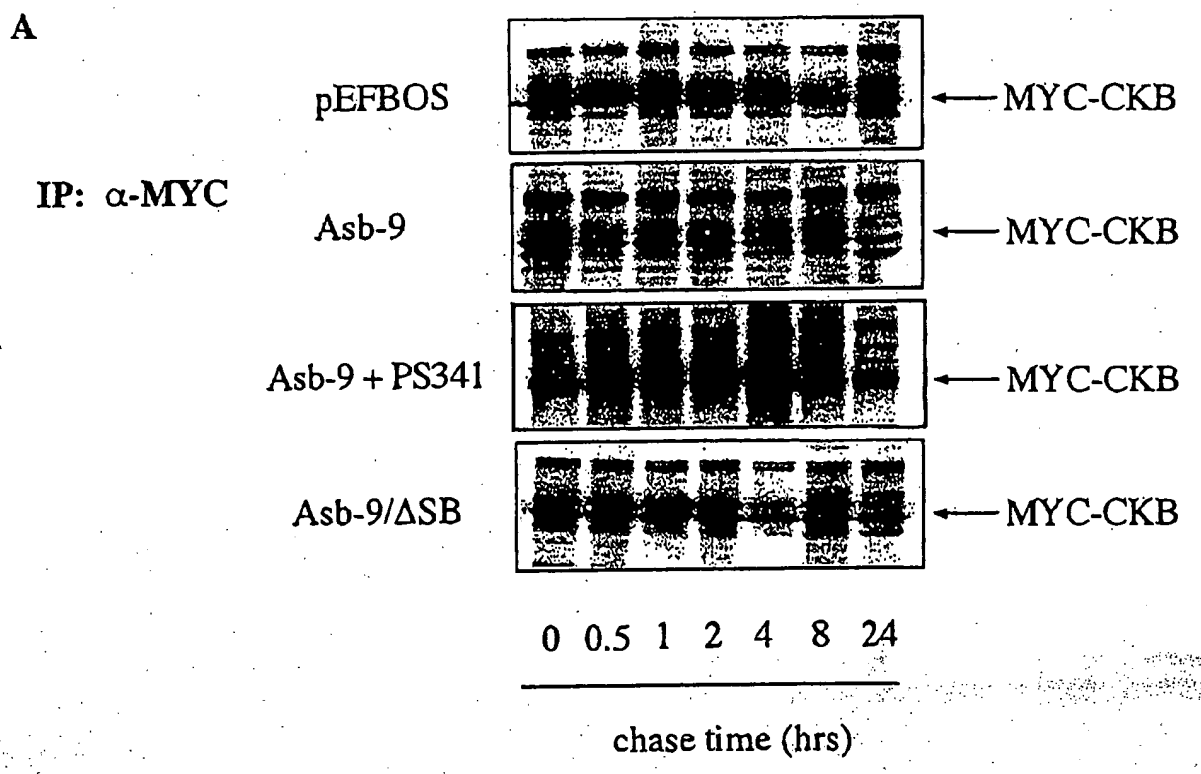

Asb-9 and pEFBOS
Asb-9/ΔSB and pEFBOS
293T
0
10ng
50ng
100ng
200ng
500ng
1.0μg
2.5μg
293T
0
10ng
50ng
100ng
200ng
500ng
1.0μg
2.5μg
kDa
41
32
A
B
C
D
E
F
W.
W
W.

Figure 6


A
IP: α-MYC
pEFBOS
Asb-9
Asb-9 + PS341
Asb-9/ΔSB
MYC-CKB
MYC-CKB
MYC-CKB
MYC-CKB
0 0.5 1 2 4 8 24
chase time (hrs)

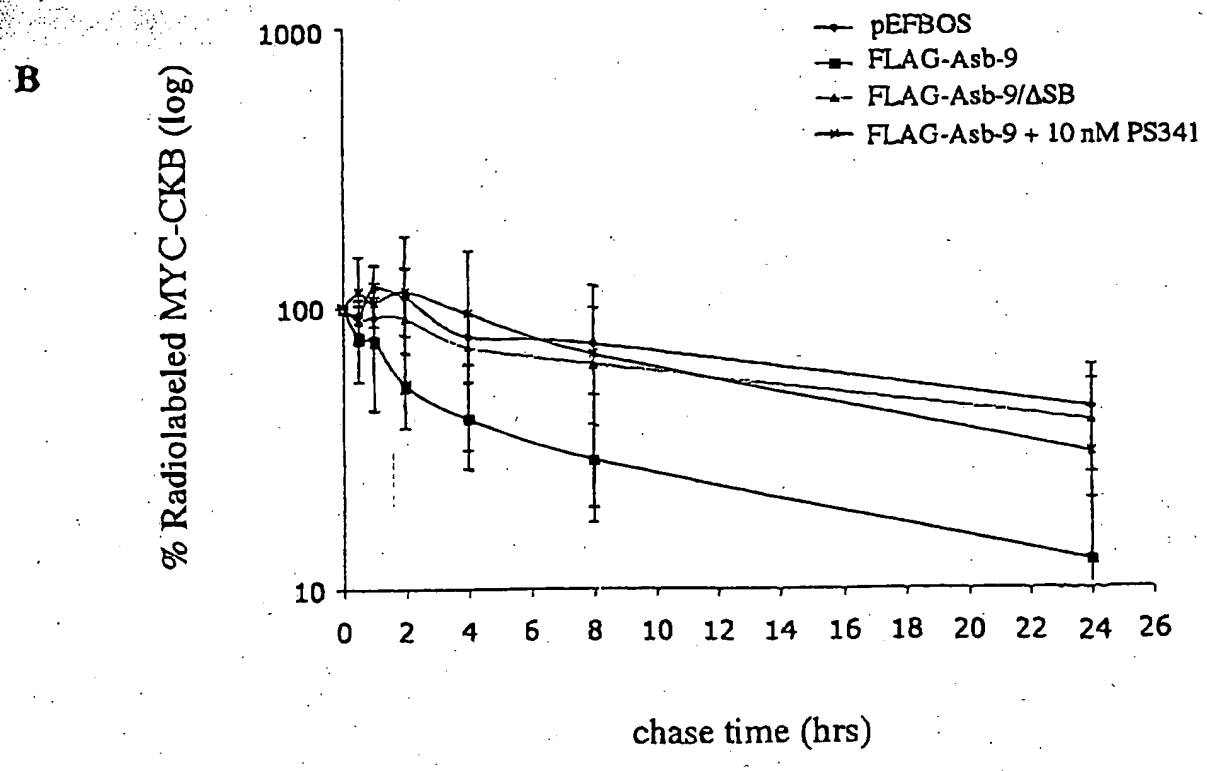
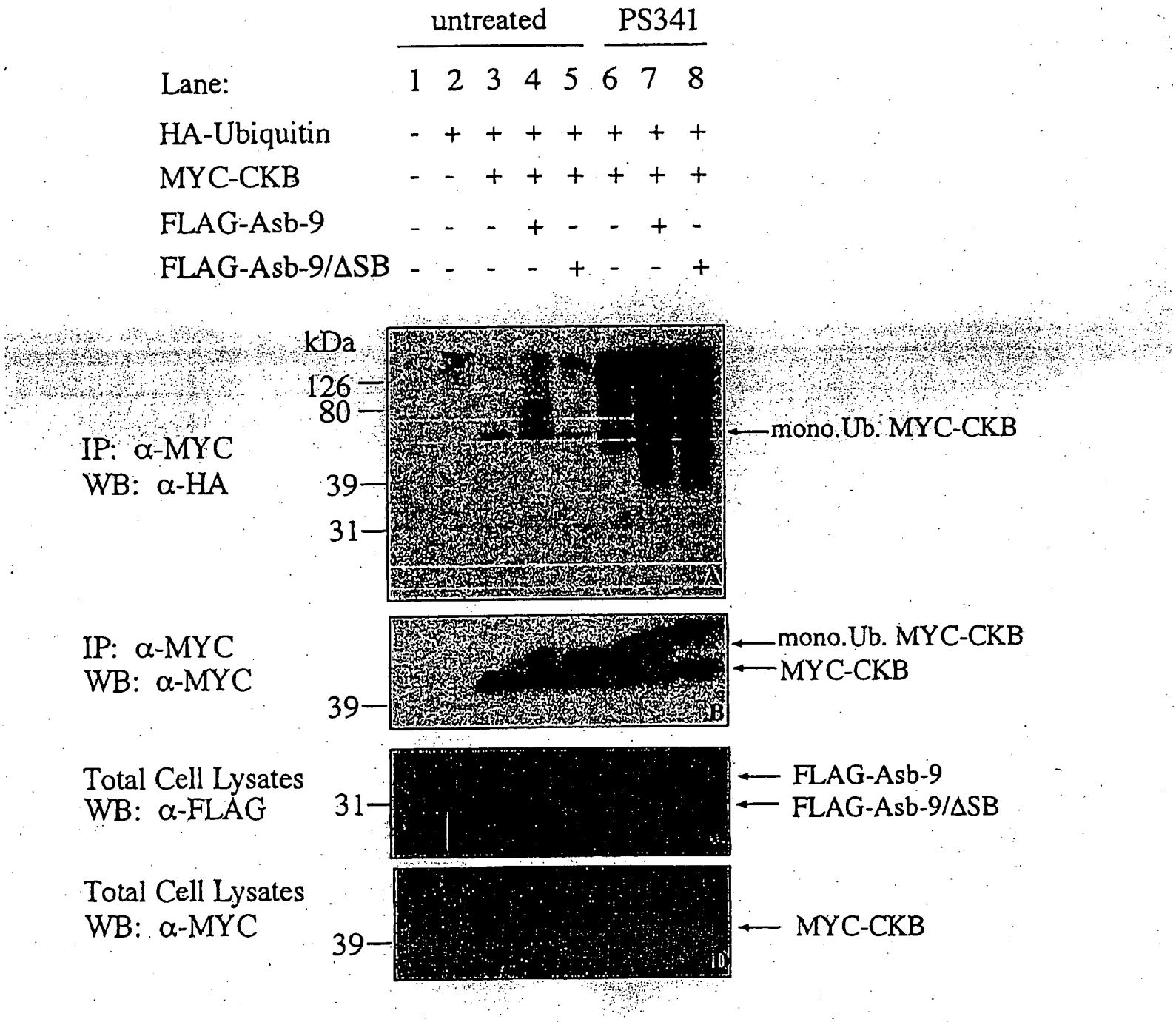

B
% Radiolabeled MYC-CKB (log)
1000
100
10
0 2 4 6 8 10 12 14 16 18 20 22 24 26
chase time (hrs)
pEFBOS
FLAG-Asb-9
FLAG-Asb-9/ΔSB
FLAG-Asb-9 + 10 nM PS341

Figure 7


untreated
PS341
Lane: 1 2 3 4 5 6 7 8
HA-Ubiquitin - + + + + + + +
MYC-CKB - - + + + + + +
FLAG-Asb-9 - - - + - - + -
FLAG-Asb-9/ΔSB - - - - + - - +
kDa
126
80
39
31
IP: α-MYC
WB: α-HA
mono.Ub. MYC-CKB
A
IP: α-MYC
WB: α-MYC
mono.Ub. MYC-CKB
MYC-CKB
39
B
Total Cell Lysates
WB: α-FLAG
31
FLAG-Asb-9
FLAG-Asb-9/ΔSB
Total Cell Lysates
WB: α-MYC
39
MYC-CKB
D